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## ILLUSTRATED REVIEW

# Advances in Clinical and Basic Science of Coagulation: Illustrated abstracts of the 9th Chapel Hill Symposium on Hemostasis

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## Abstract

This 9th Symposium on Hemostasis is an international scientific meeting held biannually in Chapel Hill, North Carolina. The meeting is in large measure the result of the close friendship between the late Dr. Harold R. Roberts of UNC Chapel Hill and Dr. Ulla Hedner of Novo Nordisk. When Novo Nordisk was developing the hemophilia therapy that would become NovoSeven, they sponsored a series of meetings to understand the basic biology and clinical applications of factor VIIa. The first meeting in Chapel Hill was held April 4-6, 2002 with Dr. Roberts as the organizer. Over the years, the conference emphasis has expanded from discussions of factor VIIa and tissue factor to additional topics in hemostasis and thrombosis. This year's meeting includes presentations by internationally renowned speakers that discuss the state-of-the-art on an array of important topics, including von Willebrand factor, engineering advances, coagulation and disease, tissue factor biology, therapeutic advances, and basic clotting factor biology. Included in this review article are illustrated abstracts provided by our speakers, which highlight the main conclusions of each invited talk. This will be the first meeting without Dr. Roberts in attendance, yet his commitment to excellent science and his focus on turning science to patient care are pervasively reflected in the presentations by our speakers.

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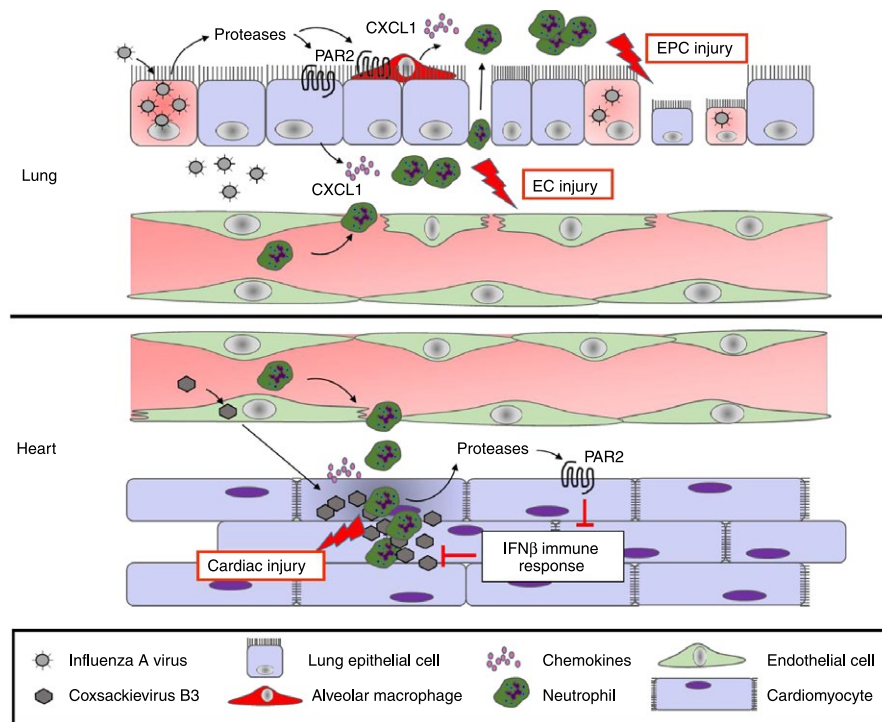
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# PAR2 in viral infections

Silvio Antoniak PhD

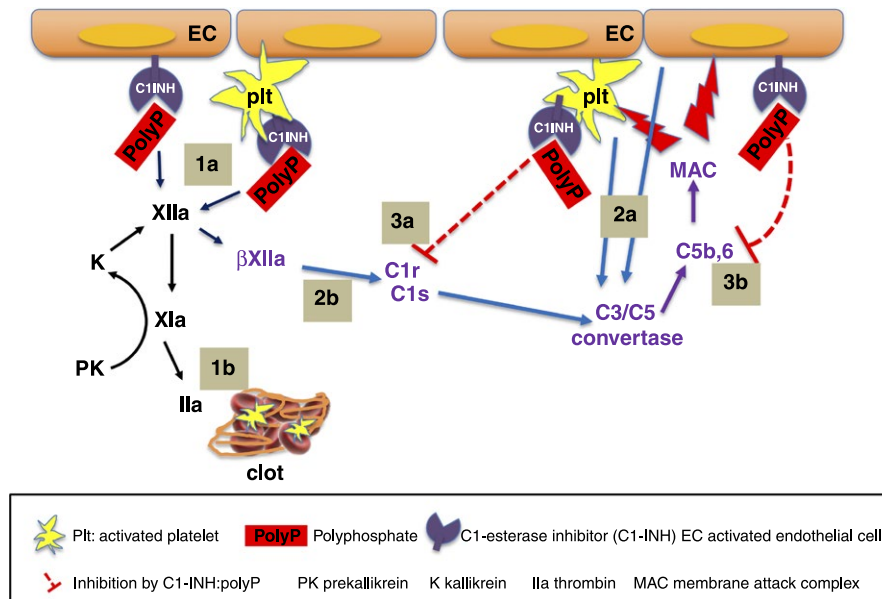


There is a known interaction between the blood coagulation and innate immune system partly mediated by protease-activated receptors (PARs).<sup>1</sup> During viral infection, pathogen and host-derived proteases including known PAR2 activators are generated. Importantly, we and others showed that PAR2 dampens antiviral interferon  $\beta$  (IFN $\beta$ ) but increases NF $\kappa$ B responses after viral infections.<sup>1-3</sup> In viral myocarditis, cardiac PAR2 inhibits IFN $\beta$  responses during Cocksackievirus B3 virus infection causing increased cardiac virus load and inflammation and subsequently increased cardiac injury.<sup>2</sup> In addition, we and others found that PAR2 increases H1N1 influenza A virus (IAV) pathology.<sup>3</sup> Recent findings suggest that myeloid cell (possibly macrophage) and lung epithelial cell PAR2 contribute to IAV pathology. Preliminary data showed that PAR2 activation leads to increased CXCL1 expression and neutrophil infiltration into the lung and subsequently causing endothelial cell (EC) and epithelial cell (EPC) injury. The current data suggest a potential therapeutic strategy by blocking the pathologic PAR2 signaling pathway to improve the outcome and severity of virus infections.

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# Polyphosphate complements coagulation

Edward M. Conway MD, PhD



In response to injury, platelets (plt) and endothelial cells (ECs) are activated, causing polyphosphate (polyP) and C1-esterase inhibitor (C1-INH) to colocalize on the cells. PolyP triggers coagulation via activation of factors XII and XI to XIIa and XIa, respectively (1a), leading to thrombin (IIa) generation and clot formation<sup>4</sup> (1b). XIIa also converts prekallikrein (PK) to kallikrein (K), which yields more XIIa and bXIIa.

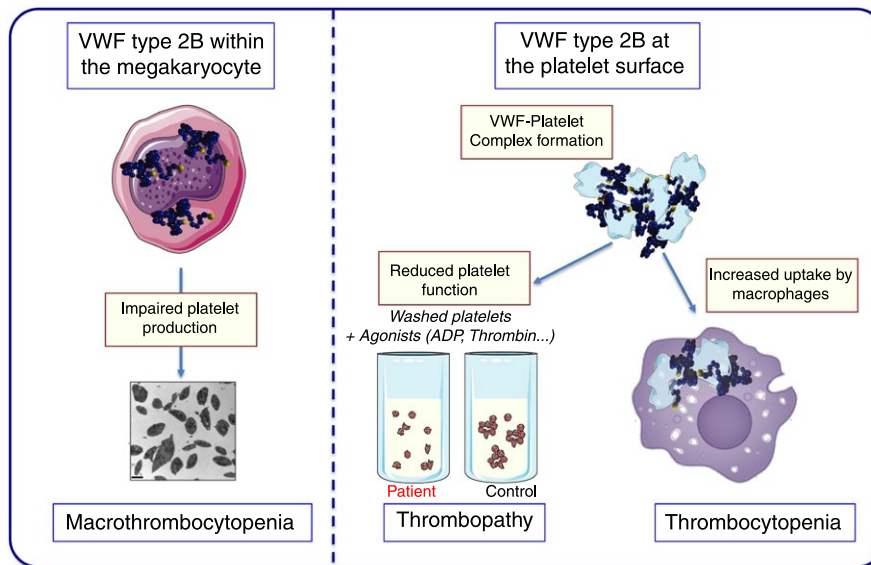
Activated platelets and endothelial cells also trigger complement activation (2a), promoting assembly of C3 and C5 convertases, which lead to formation of C5b,6 and the C5b-9 membrane attack complex (MAC). bFXIIa also contributes by activating complement factors C1r and C1s (2b).

If unchecked, complement activation results in autoimmune destruction of the activated endothelial cells and platelets, abrogating their procoagulant functions. PolyP prevents this by potentiating the inhibitory properties of C1-INH, such that it neutralizes C1r and C1s (3a), and destabilizes C5b,6<sup>6</sup> (3b), reducing MAC formation.

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# Von Willebrand disease type 2B: an ever surprising condition

Cécile V. Denis PhD

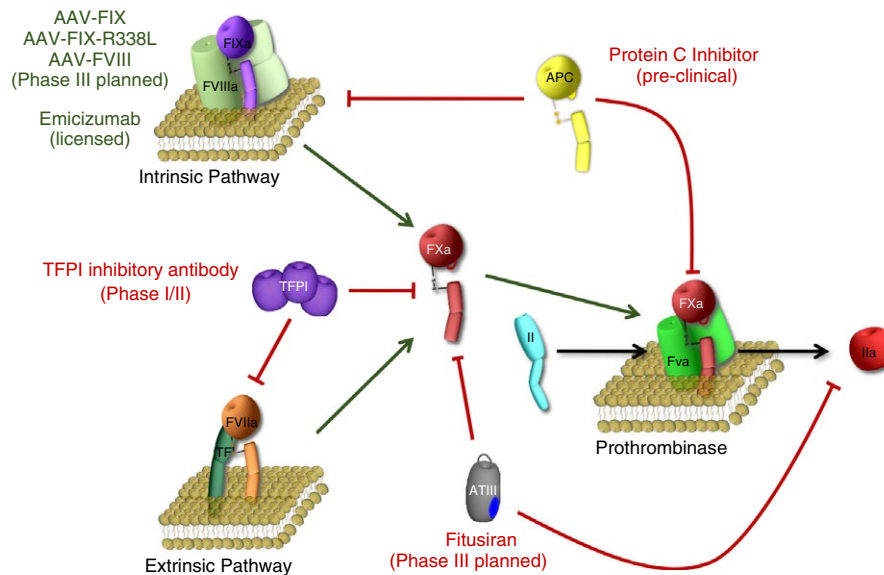


Von Willebrand disease type 2B (vWD-type 2B) is a counterintuitive pathological condition in which gain-of-function mutations in the plasma protein von Willebrand factor (VWF) lead to a bleeding rather than a prothrombotic phenotype. For the most severe mutations, underlying molecular mechanisms involve: (1) defective platelet function due to impaired signaling leading to lack of activation of  $\alpha\text{IIb}\beta 3$  integrin<sup>7</sup> and (2) thrombocytopenia of various severity. This thrombocytopenia originates from a central production defect<sup>8</sup> as well as from an increased clearance of platelets from the circulation.<sup>9</sup> How mutations in VWF can induce such a variety of cellular effects is currently under investigation. Interestingly, strategies aiming to restore a normal platelet count such as thrombopoietin receptor agonists or LIM kinase inhibitors may be beneficial in some patients with vWD-type 2B.

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# Novel hemophilia therapeutics

Lindsey A. George MD

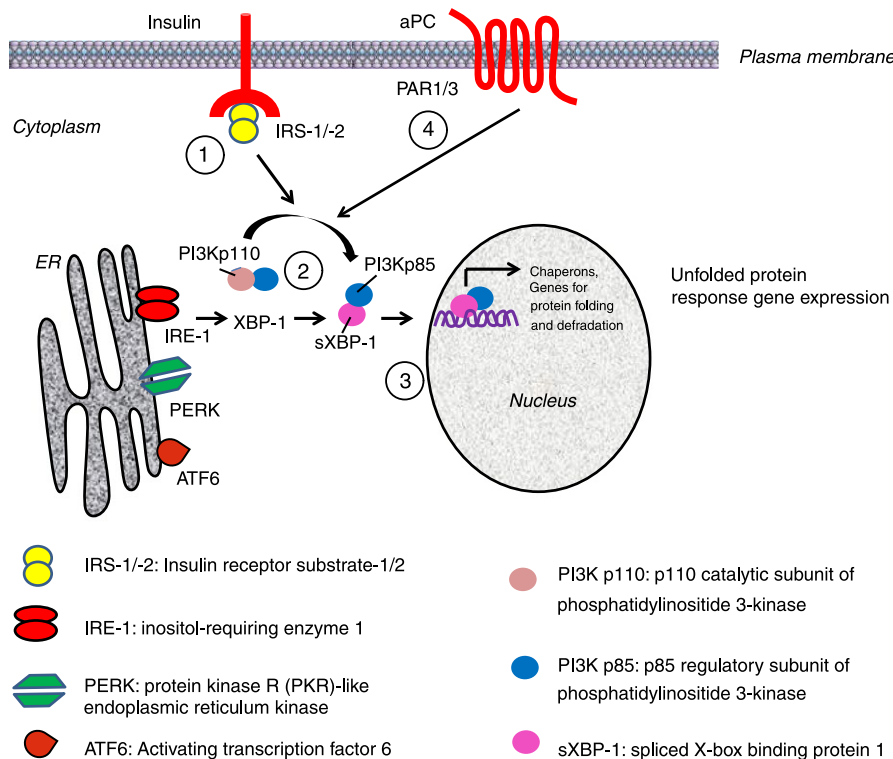


Novel therapeutics for hemophilia are poised to alter the current treatment paradigm. Traditional factor-replacement has normalized the life expectancy of hemophilia patients. However, several aspects remain suboptimal, including the need for recurrent intravenous administration and thus significant planning, and the risk of alloantibody formation. Current novel therapeutics include successful early-phase adeno-associated virus-mediated factor VIII and IX gene transfer, a FVIII-mimetic antibody (emicizumab) and strategies to disrupt natural anticoagulants, including tissue factor pathway inhibitor (TFPI) antibodies, an activated protein C (APC) serpin, and RNAi knock-down of antithrombin III (ATIII) with fitusiran.<sup>10–15</sup> Importantly, these new agents can be administered subcutaneously and/or at a reduced frequency. However, thrombotic complications and deaths reported in clinical trials with emicizumab and fitusiran highlight the importance of bridging science with clinical care to ensure safe adaptation. Nonetheless, this expanding therapeutic repertoire provides an optimistic outlook for the future of hemophilia care.

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# Cross-talk of insulin and aPC signaling in the kidney

Berend Isermann MD



Insulin resistance and diabetes mellitus are associated with impaired renal function. Expression of the insulin receptor in the kidney is established,<sup>16</sup> but the mechanism through which insulin resistance contributes to diabetic nephropathy remains unknown. We demonstrated that insulin signaling (1) induces dissociation of the regulatory p85-subunits from the endoplasmic reticulum (ER)-dependent transcription factor spliced XBP-1 (sXBP1, 2), promoting its nuclear translocation (3).<sup>17</sup> This effect, which conveys protective aspects of ER-signaling, is lost in insulin resistance, promoting diabetic nephropathy. Activated protein C (aPC) signaling (4) via protease activated receptors 1 and 3 (PAR1/3) restores sXBP1-p85 dissociation (2) and sXBP1 nuclear translocation (3), maintaining kidney function.<sup>18</sup> In vivo, aPC restores ER-function and protects mice lacking the insulin receptor from aggravated diabetic nephropathy. This unravels a new function of coagulation proteases, compensating for defective insulin signaling, and alludes to new therapeutic options in diabetic nephropathy.

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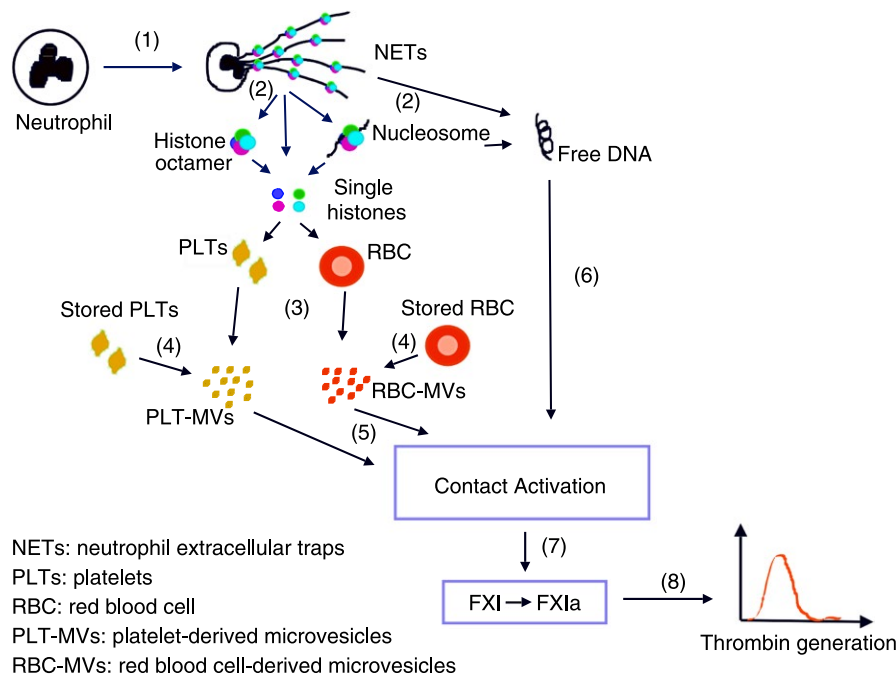
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# Cellular products and contact activation

Nigel S. Key MD

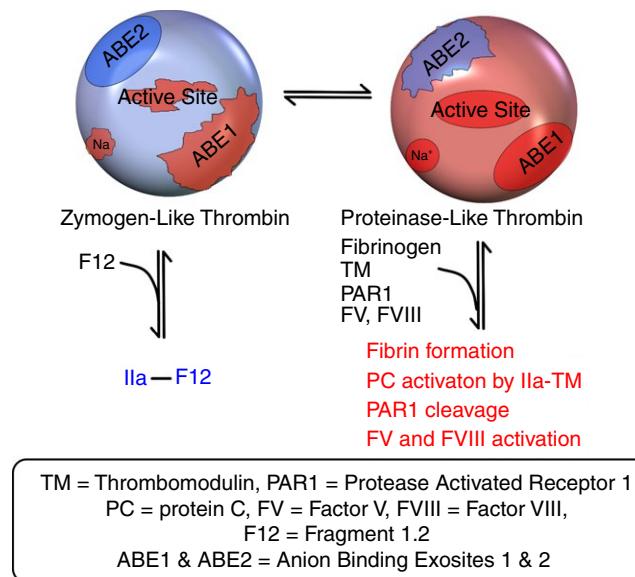


Cell damage and/or death occur during several pathological processes that are associated with an increased risk of thrombosis. Neutrophil extracellular traps (NETs), which are composed of a backbone of chromatin, can be expelled by activated neutrophils (1). NETs can be degraded into their component molecular structures including nucleosomes, histone octamers, single histones and/or free DNA (2). Incubation of single histones (H3 and H4) with platelets or red blood cells (RBCs) induces microvesicle (MV) release from both of these cell types (3). Platelet- and RBC-derived MVs are also generated in red cell and platelet units for transfusion during blood bank storage (4). RBC-MVs and PLT-MVs activate the contact system (5). Unlike histones, nucleosomes or intact NETs, free DNA directly triggers contact activation (6). The activated form of factor XII (FXIIa) is generated following contact activation. FXIIa may promote activation of factor XI (7), which mediates downstream thrombin generation (8).

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# Specificity and function in thrombin and the products of prothrombin activation

Sriram Krishnaswamy PhD



The cleavage of prothrombin to thrombin is required for fibrin formation and platelet activation. Thrombin also plays both positive and negative regulatory roles in coagulation because of its pan-specific action on multiple coagulation proteins. These different, and sometimes opposing functions arise from the binding of ligands and substrates to two exosites (ABE1 and ABE2) on the proteinase. Thrombin employs ABE1 to act on numerous substrates and to bind different ligands. The authentic protein ligand for ABE2 is the N-terminal propeptide of prothrombin released upon thrombin formation or covalently retained in the intermediate, meizothrombin.<sup>21</sup> Competitive binding interactions between common ligands for any one exosite and reciprocal allosteric effects of ligand binding to the two exosites differentially impact the range of thrombin functions.<sup>22,23</sup> Both exosite binding and allosteric transitions, arising from these binding steps, regulate the essential roles played by thrombin and its precursors in coagulation.

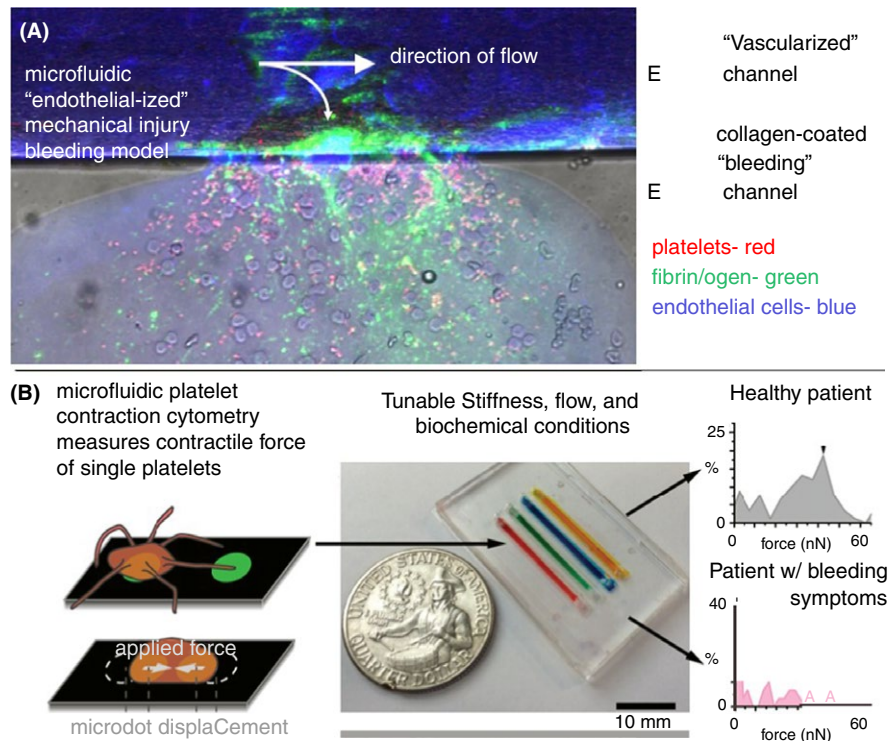
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# Next generation microfluidic devices to investigate hemostasis

Wilbur A. Lam MD, PhD



Research from the last decade has demonstrated microfluidic devices, which are developed from microchip-based technologies, to be ideal tools for quantitatively analyzing clot formation, typically in a thrombotic context.<sup>24,25</sup> More recently, several groups including our own have begun developing microfluidic systems that model bleeding and function as quantitative research-enabling tools to study the hemostatic process in vitro. These next generation microfluidic devices recapitulate physiologic biological, biochemical, and/or biophysical aspects of hemostasis that existing in vitro assays are incapable of. For example, a microfluidic bleeding model comprising an "endothelialized" microchannel coupled with a microengineered pneumatic valve that induces a mechanical vascular "injury" allows for visualization of hemostatic plug formation and measurement of an "in vitro bleeding time" (Section A). In addition, high-throughput contractile force measurements of individual platelets enabled by microfluidic platelet contraction cytometry may serve as a new platelet function test for platelet-based bleeding disorders (Section B).<sup>26</sup>

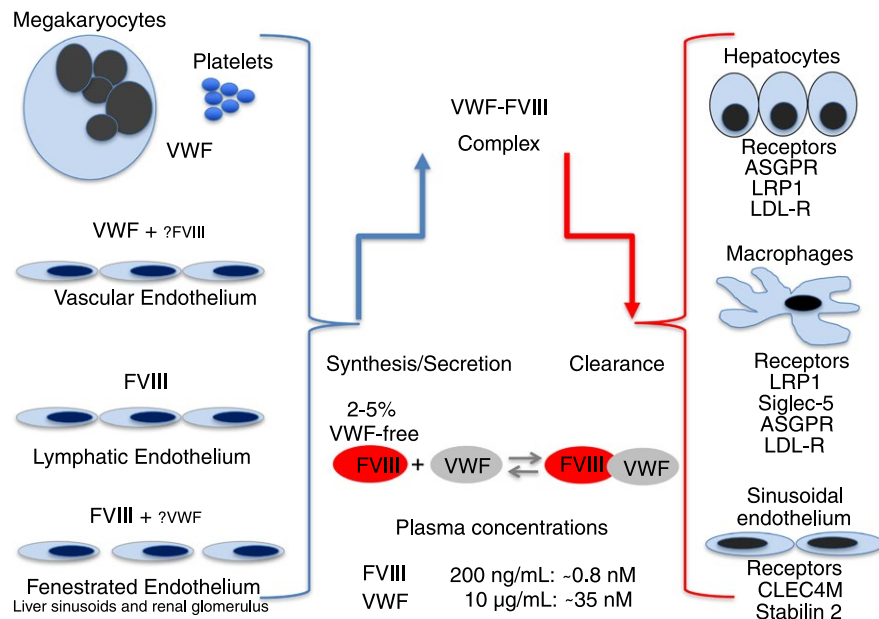
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# Factor VIII and von Willebrand factor: a complex relationship

David Lillicrap MD



Deficiencies or dysfunction of the procoagulant cofactor, factor VIII (FVIII), or the adhesive protein, von Willebrand factor (VWF), represent the most common inherited bleeding disorders in humans. These proteins have inter-related life cycles and circulate in the plasma in a tight noncovalent complex.<sup>27</sup> VWF plays a dominant role in this interaction. After many years of debate, it appears that both proteins are synthesized predominantly in endothelial cells, although the subtypes of endothelium that are involved and the fact that most cells express only one of the two proteins are features that require more study.<sup>28</sup> In the plasma, VWF protects FVIII from premature proteolytic cleavage by activated protein C and acts to guide FVIII to the site of vessel wall damage. Ultimately, both FVIII and VWF are cleared from the circulation by macrophages, hepatocytes and sinusoidal endothelial cells in the spleen and liver through interactions involving several lectin and scavenger receptors.<sup>29</sup>

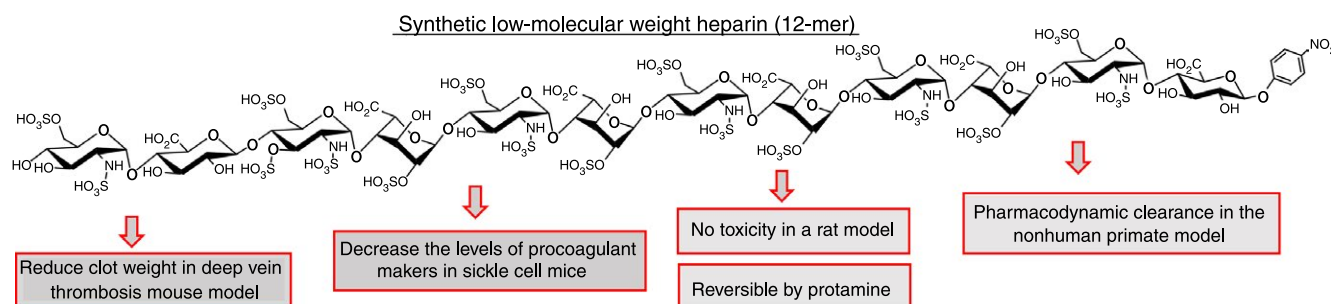
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# Developing an enzyme-based approach to prepare synthetic low-molecular-weight heparin

Jian Liu PhD

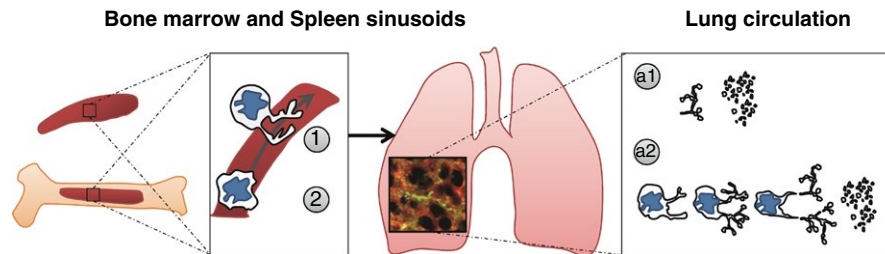


Low-molecular-weight heparin (LMWH) is clinically used to treat clotting disorders. As an animal-sourced product, LMWH's supply chain reliability is a concern for regulatory agencies. Here, we demonstrate the synthesis of heparin dodecasaccharides (12-mers) at gram-scale. In vitro and ex vivo experiments demonstrate that the anticoagulant activity of the 12-mers could be reversed using protamine. The 12-mer reduced the size of blood clot in the mouse model of deep vein thrombosis, and attenuated the procoagulant markers in the mouse model of sickle-cell disease. The 12-mer was examined in a nonhuman primate model to determine the pharmacodynamics parameters. A 7-day toxicity study in a rat model showed no toxic effects. The data suggest that a synthetic homogeneous oligosaccharide can replace animal-sourced LMWHs.

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# Platelet biogenesis in the lung circulation

Mark R. Looney MD

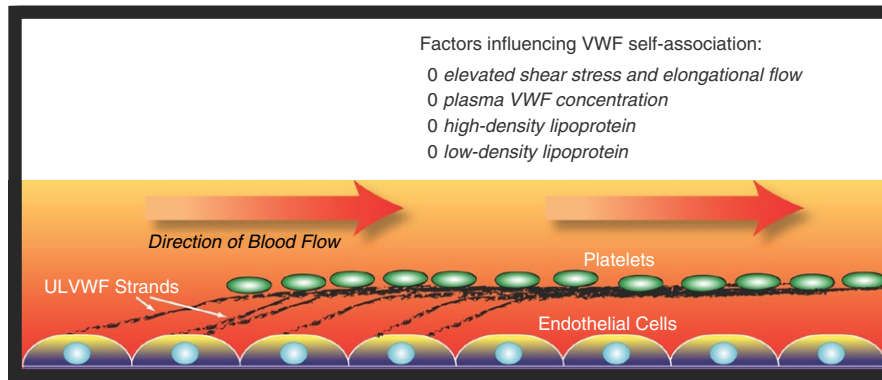


Platelets are indispensable in hemostasis, thrombosis, and immune responses. In humans, billions of platelets are produced each day from megakaryocytes, however the mechanisms of mature platelet production are incompletely understood. Historical data indicate that the lung may be a site of platelet biogenesis. Using intravital imaging in mice,<sup>31</sup> megakaryocytes were visualized releasing platelets in the lung circulation, and this process was responsible for approximately half of the mature platelet production in mice.<sup>32</sup> The mechanisms by which the lung circulation facilitates platelet biogenesis is a novel area of investigation for understanding platelet production defects. The lung may be a suitable bioreactor for the production of mature platelets from in vitro-derived megakaryocytes.<sup>33</sup> The lung also contains "resident" megakaryocytes and hematopoietic precursors, the latter being capable of migrating to the bone marrow to participate in the production of platelets and other mature hematopoietic lineages.

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# Regulation of von Willebrand factor function at the level of self-association

José A. López MD



Von Willebrand factor is the largest protein in blood plasma and has multiple roles in hemostasis, the best known of which is to mediate the attachment of platelets to the vessel wall, usually at sites of injury. In pathological states, VWF can remain attached to the vessel wall and allow platelets to attach to intact vascular endothelium. The ability of VWF to carry out its adhesive function is regulated by a number of factors, including the concentration of the protein, its multimeric structure, and the degree to which it is proteolyzed by the plasma protease ADAMTS13. Less appreciated as important for VWF function is its ability to self-associate, taking already very long molecules and assembling them into strands of potentially prodigious sizes (see Figure).<sup>34,35</sup> VWF self-association is itself regulated by a variety of factors, including concentration, shear stress and elongational flow/proteolysis, and high density lipoproteins.<sup>36</sup>

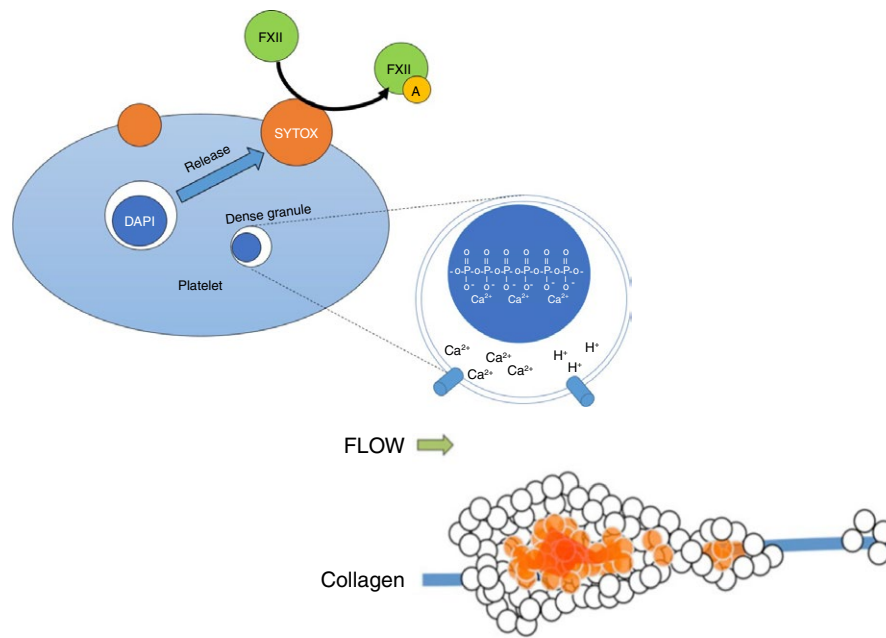
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# Platelet polyphosphate and factor XII (nature's way to engineer nanoparticles)

Coen Maas PhD



The contact system contributes to coagulation *in vitro*, but contact factor deficiencies do not cause bleeding. Remarkably, the contact system has a pronounced role in mouse thrombosis models. Here, platelet polyphosphate was proposed as the endogenous trigger for Factor XII (FXII) activation.<sup>37,38</sup> This is surprising as short platelet polyphosphate polymers have limited potential for contact activation.

Contact system activators in clotting reagents are usually fine particulates. We found that platelets store and release polyphosphate in a comparable state as polyphosphate nanoparticles, which can be tracked with DAPI (membrane-permeant) and SYTOX (membrane-impermeant).<sup>39</sup> Polyphosphate nanoparticles remain on the platelet surface under flow, and can be isolated from platelet lysates. Platelet-sized polyphosphate efficiently triggers contact system activation as a nanoparticle state, but much less as a soluble polymer.

Extracellular polyphosphate is concentrated in the core of the thrombus, but not in the shell. This favors the concept that the contact system contributes to thrombus stability: once a thrombus is physically “threatened” and plasma reaches the thrombus core, FXII-driven thrombin formation is triggered to ensure thrombus stability.

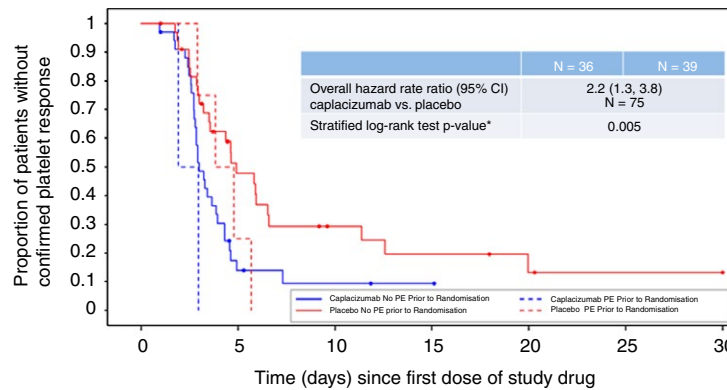
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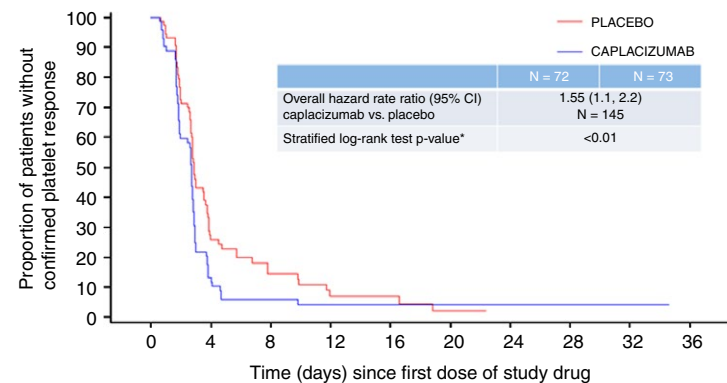
# New treatment options in TTP

Flora Peyvandi MD, PhD

TITAN  
caplacizumab  
Phase II trial



HERCULES  
caplacizumab  
phase III trial



Thrombotic thrombocytopenic purpura (TTP) is a rare, acute-onset disease caused by the congenital or acquired deficiency of ADAMTS13, leading to microvascular thrombosis and ischemic organ damage. The mainstays of acute TTP treatment are plasma infusion (hereditary TTP) and plasma-exchange in conjunction with immunosuppressive therapy (acquired TTP). Despite a dramatic improvement in TTP diagnosis and treatment, mortality (10%), exacerbation (30%), major thrombotic complications, refractoriness to standard therapy and long-term complications are still major issues demanding novel therapies. Recombinant ADAMTS13<sup>40</sup> and caplacizumab,<sup>41,42</sup> a nanobody against the platelet-binding domain of VWF, recently completed phase I (clinicaltrials.gov NCT02216084) and III (HERCULES trial, clinicaltrials.gov NCT02553317) clinical trials for the treatment of hereditary and acquired TTP, respectively. Recombinant ADAMTS13 was safe, well-tolerated, with pharmacokinetic parameters comparable with those from plasma infusion studies.<sup>40</sup> Caplacizumab met primary and key secondary endpoints in HERCULES trial,<sup>42</sup> confirming previous phase II results from the TITAN trial.<sup>41</sup>

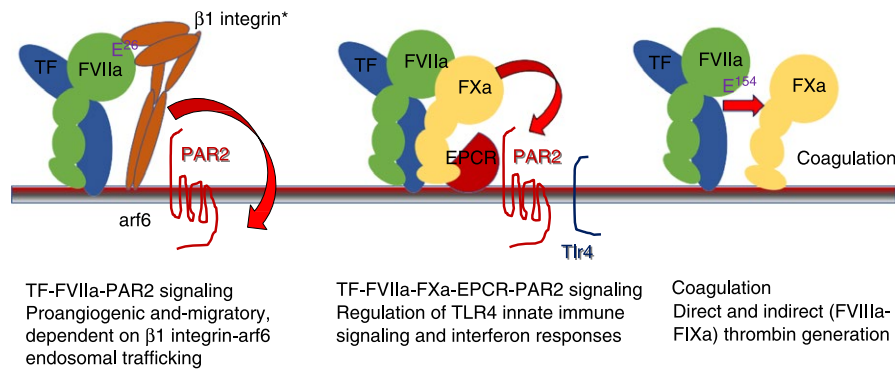
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# TF control of PAR2 signaling

Wolfram Ruf MD



Protease activated receptor (PAR) 2 is the target for upstream coagulation proteases, FVIIa and FXa, and cleaved during TF-initiated coagulation. We have identified crucial residues in the FVIIa protease domain that regulate ligand binding and thereby direct the biological functions of the TF-FVIIa complex in hemostasis and cell signaling. The integrin binding motif (KGE) residue FVII E<sup>26</sup> promotes TF-FVIIa complex formation with active integrin  $\beta 1$  and couples TF to integrin-arf6 recycling required for endosomal pro-angiogenic and pro-migratory PAR2 signaling<sup>43</sup>. In the same region of the FVIIa protease domain, the allosteric switch residue FVIIa E<sup>154</sup> controls the release of nascent FXa product required for TF prothrombotic activity. In contrast, stability of the TF-FVIIa-FXa ternary complex favors hemostatic cofactor FVIII activation<sup>44</sup> and EPCR-dependent cleavage of PAR2 by FXa that is crucial for toll-like receptor (TLR) 4 innate immune signaling, specifically the induction of interferon-regulated genes<sup>45</sup>. Mutants of PAR2 with resistance to selective coagulation proteases and cell type-specific coagulation factor deficiencies begin to delineate the distinct contributions of TF signaling complexes in the regulation of immune responses and cancer progression.

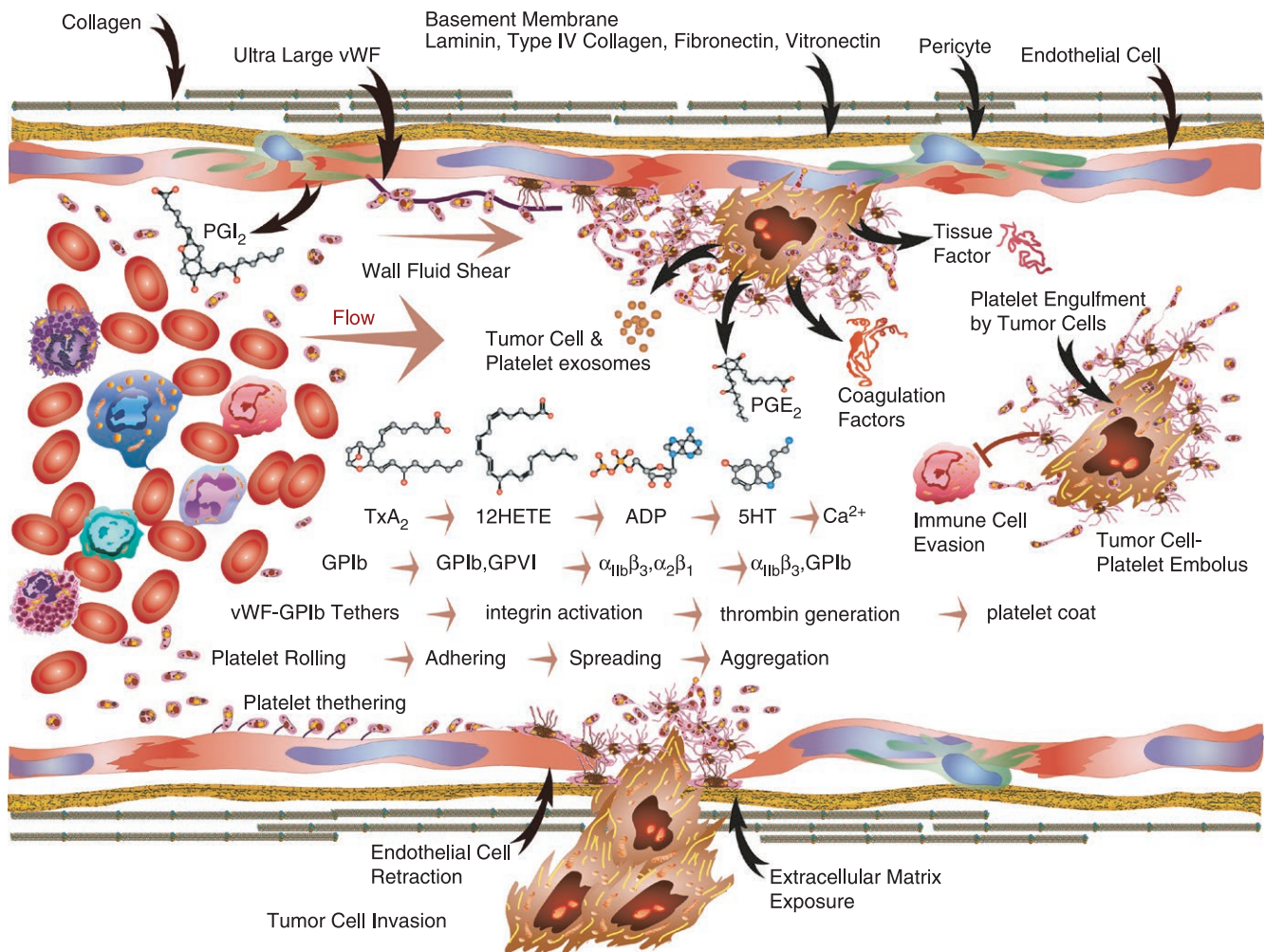
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# The platelet lifeline to cancer

Anil K. Sood MD



Platelets are well known to play important roles in hemostasis and wound remodeling and healing. However, the bidirectional interplay between platelets and cancer is increasingly recognized for its impact on tumor biology and patient outcomes. Thrombocytosis, resulting from tumor-derived factors, occurs with varying frequency in cancer patients and is associated with adverse clinical outcomes.<sup>46</sup> Biologically, cancer is often considered analogous to a non-healing or chronic wound. There is growing evidence that platelets play key roles in many steps of tumor development and progression including proliferation, extravasation, survival, and metastasis.<sup>47</sup> Moreover, platelets may actively infiltrate into the tumor microenvironment and facilitate evasion from cancer therapies.<sup>48</sup> Understanding these complex interactions related to the roles of platelets in tumor biology offer new opportunities for reducing thrombosis, blocking tumor growth, and ultimately improving patient outcomes.

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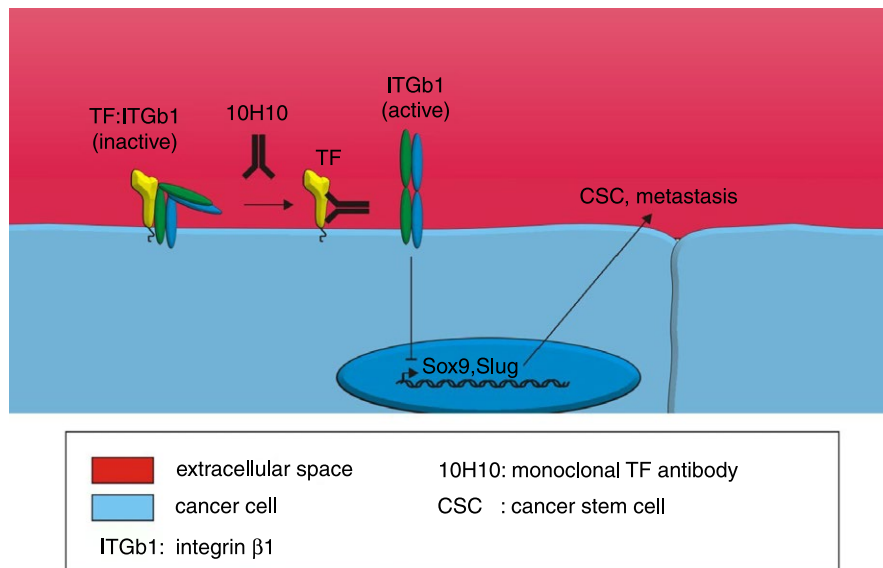
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# TF and cancer: blocking tissue factor signaling in breast cancer inhibits tumor metastasis

Henri H. Versteeg PhD



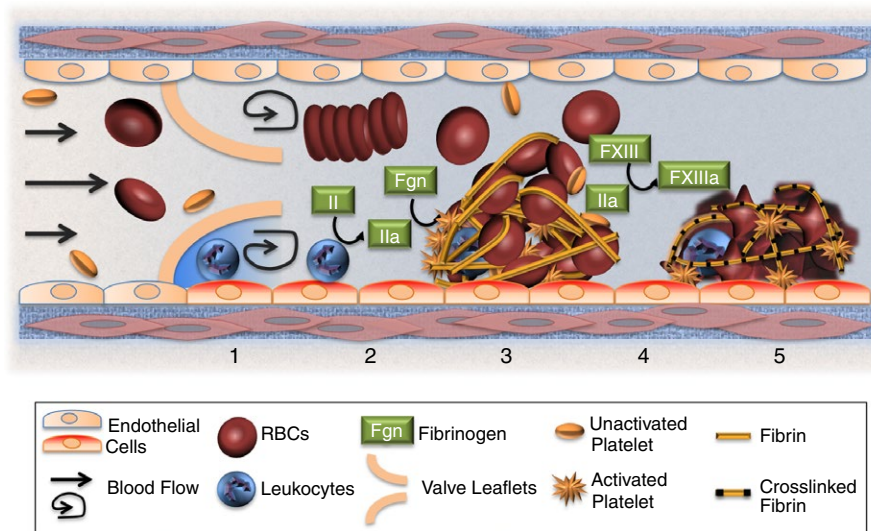
Tissue factor (TF) promotes breast cancer metastasis by activating coagulant pathways,<sup>50,51</sup> but the role of TF signaling in metastasis has never been addressed. We show that an antibody against TF that inhibits signaling uncouples TF from integrin  $\alpha$ 3 $\beta$ 1 and leads to a net cellular increase in active integrin  $\beta$ 1. In vitro, TF inhibition leads to a reduction in cancer stem cells (CSCs) and CSC effector molecules such as Sox9 and Slug. Such a link between TF and CSC is supported by an association between TF expression and the CSC marker aldehyde dehydrogenase 1 (ALDH1) in tumor specimens from 574 breast cancer patients. In vivo we show that inhibition of TF signaling inhibits metastasis 10-fold when given in a scheme that has little effect on primary tumor growth. Thus, TF signaling inhibition leads to a reduced CSC transcriptional program, a reduction in primary tumor-resident cancer stem cells and a subsequent reduction in metastasis.

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# Fibrinogen and factor XIII in venous thrombosis

Alisa S. Wolberg PhD

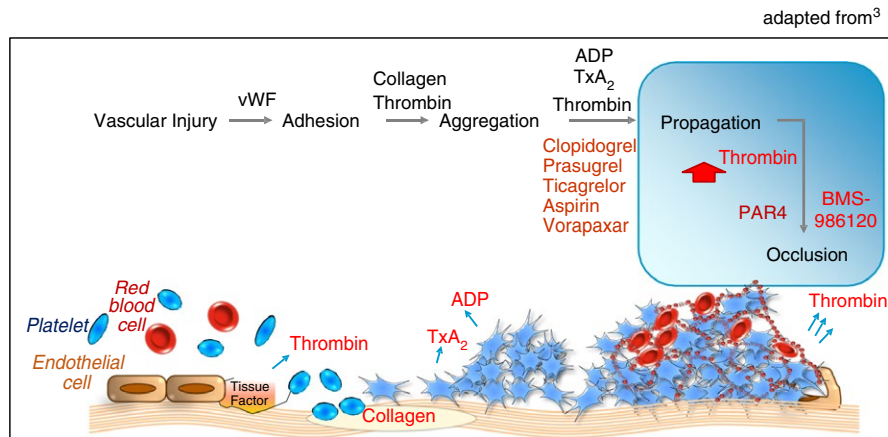


Venous thrombosis is a leading cause of death and disability worldwide. Venous thrombi typically originate in hypoxic venous valve pockets and areas of reduced blood flow (stasis), where activated endothelium expresses cell adhesion molecules that engage leukocytes at the vessel wall (1). Procoagulant endothelium and leukocytes support thrombin (IIa) generation (2), which leads to conversion of fibrinogen to fibrin and formation of a fibrin network that entraps red blood cells (RBCs) (3). Thrombin also converts factor XIII (FXIII) to an active transglutaminase (FXIIIa) (4), which crosslinks fibrin and also crosslinks plasma proteins to fibrin. Crosslinked fibrin retains RBCs in clots during platelet-mediated clot contraction (5),<sup>52,53</sup> and crosslinked  $\alpha_2$ -antiplasmin increases clot resistance to fibrinolysis. Although FXIII is found in both cells and plasma, plasma FXIII is the major contributor to fibrin crosslinking and RBC retention, and mediates thrombus size in a dose-dependent manner.<sup>54</sup> FXIII(a) may be a therapeutic target for reducing venous thrombosis.

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# Thrombin receptor PAR4: new target for antiplatelet therapy

Pancras C. Wong PhD



Thrombin is a potent agonist that induces platelet aggregation via the G-protein coupled protease-activated receptors (PAR), PAR1 and PAR4.<sup>55</sup> PAR1 is a target of antiplatelet therapy in the clinic, but PAR1 inhibitors have a narrow therapeutic window when given on top of standard-of-care antiplatelet agents. We hypothesized that initial platelet responses to low thrombin concentrations, mediated by PAR1, are important for hemostasis, whereas sustained platelet responses to high thrombin concentrations, mediated by PAR4, are critical for thrombus propagation and thrombosis. To validate PAR4 as a safer antiplatelet drug target, we developed the highly selective small-molecule compound inhibitor of PAR4, BMS-986120. BMS-986120 is an orally-active drug with strong antithrombotic activity and low bleeding liability in cynomolgus monkeys. It demonstrates a wider therapeutic window than the standard antiplatelet agent clopidogrel. These preclinical findings suggest that PAR4 is an effective and safe antiplatelet drug target.<sup>56</sup>

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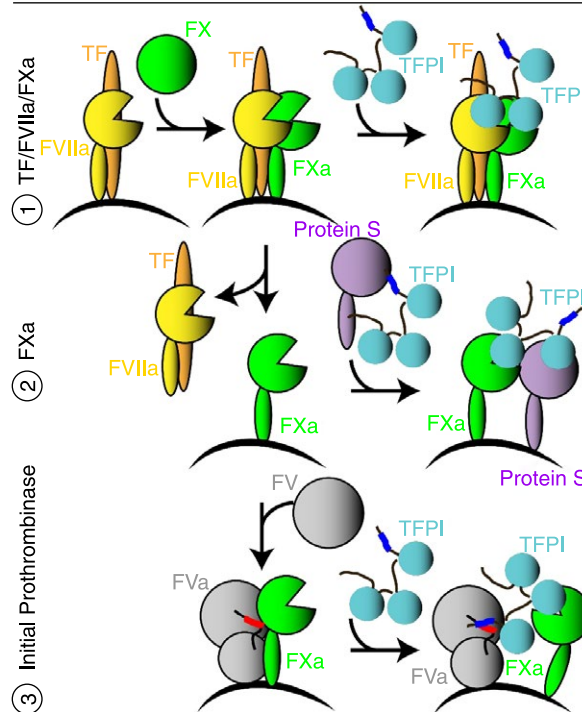
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# Tissue factor-independent inhibition of thrombin generation by TFPI $\alpha$

Jeremy P. Wood PhD

TFPI $\alpha$  Inhibits the First Three Steps of the Tissue Factor Pathway



The tissue factor (TF) pathway of thrombin generation, which commences when the TF/factor VIIa (FVIIa) complex activates the serine protease factor Xa (FXa), is inhibited by TF pathway inhibitor alpha (TFPI $\alpha$ ) at three steps. First, two inhibitory domains of TFPI $\alpha$  (K1, K2) block FXa activation by TF/FVIIa. Second, TFPI $\alpha$  also inhibits the generated FXa by blocking its active site with K2. The cofactor protein S promotes and stabilizes this interaction.<sup>58</sup> Third, TFPI $\alpha$  inhibits prothrombinase, the thrombin-producing complex of FXa and factor Va (FVa), during the initiation of thrombin generation.<sup>59</sup> This inhibition requires that K2 binds the FXa active site and that the TFPI $\alpha$  basic C-terminus binds both a regulatory acidic region present in FXa-activated and platelet-released forms of FVa and the FVa heavy chain, an interaction which displaces FXa.<sup>59,60</sup> Through these TF-dependent and -independent activities, TFPI $\alpha$  establishes an activation threshold for the initiation of coagulation.

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